PURINES AND PTERIDINES FROM THE REFLECTING PIGMENT OF THE ARTHROPOD RETINA[1]

L. H. KLEINHOLZ

The Biological Laboratories, Reed College, Portland, Oregon; and Marine Biological Laboratory, Woods Hole, Mass.

Three sets of pigments are generally found in the crustacean retina, where they may undergo photomechanical movements under control of neurosecretory hormones (Kleinholz, 1936; Welsh, 1939; Brown *et al.*, 1952, 1953). The distal retinal pigment and the so-called proximal pigment of the retinular cells are dark pigments, presumed to be melanins or ommochromes, although few studies have been made of their chemical nature. The retinal reflecting pigment has been called guanine, but this identification has been based more on the analogy with the tapetal pigment occurring in the eyes of some vertebrates than on chemical study (Welsh, 1932; Kleinholz, 1936). I attempted, a score of years ago, to examine the chemical nature of this reflecting pigment of the crustacean retina, but, beyond gathering some information on solubility properties, efforts toward more specific characterization proved abortive because of limited amounts of available material.

Development within the past decade of techniques for isolation and examination of small amounts of biological material prompted a renewed attempt to identify this reflecting material. This initial study, part of which has been reported in preliminary form (Kleinholz, 1955), was done on the lobster, *Homarus americanus*, and the chelicerate, *Limulus polyphemus*.

Methods

Eyestalks of *Homarus* were usually removed before the rest of the animal was turned to other purposes. The eyes of *Limulus*, together with adjacent tissue, were excised from animals immobilized by bleeding. Immediately after removal the eyes were placed in 95% ethanol for 2 to 4 days for hardening, after which the retinas of *Homarus* were cut from the stalks while, in *Limulus*, the extraneous tissue was dissected away from the eye. The ethanol was changed frequently until no more color was leached from the retinas.

Retinal reflecting pigment in *Homarus* does not undergo photomechanical changes and occurs as a compact layer distal to the fenestrated basement membrane, as well as in substantial deposits proximal to this membrane (Fig. 1). Initially, the reflecting layer was exposed by removing and discarding these proximal deposits and adjacent tissue; material from the reflecting layer was then scraped free in ethanol and concentrated by centrifugation. After it was found that the chromatographic results were qualitatively the same, these deposits of reflecting pigment

[1] These studies were made possible by a grant-in-aid from the American Academy of Arts and Sciences, as well as by grants from the National Science Foundation.

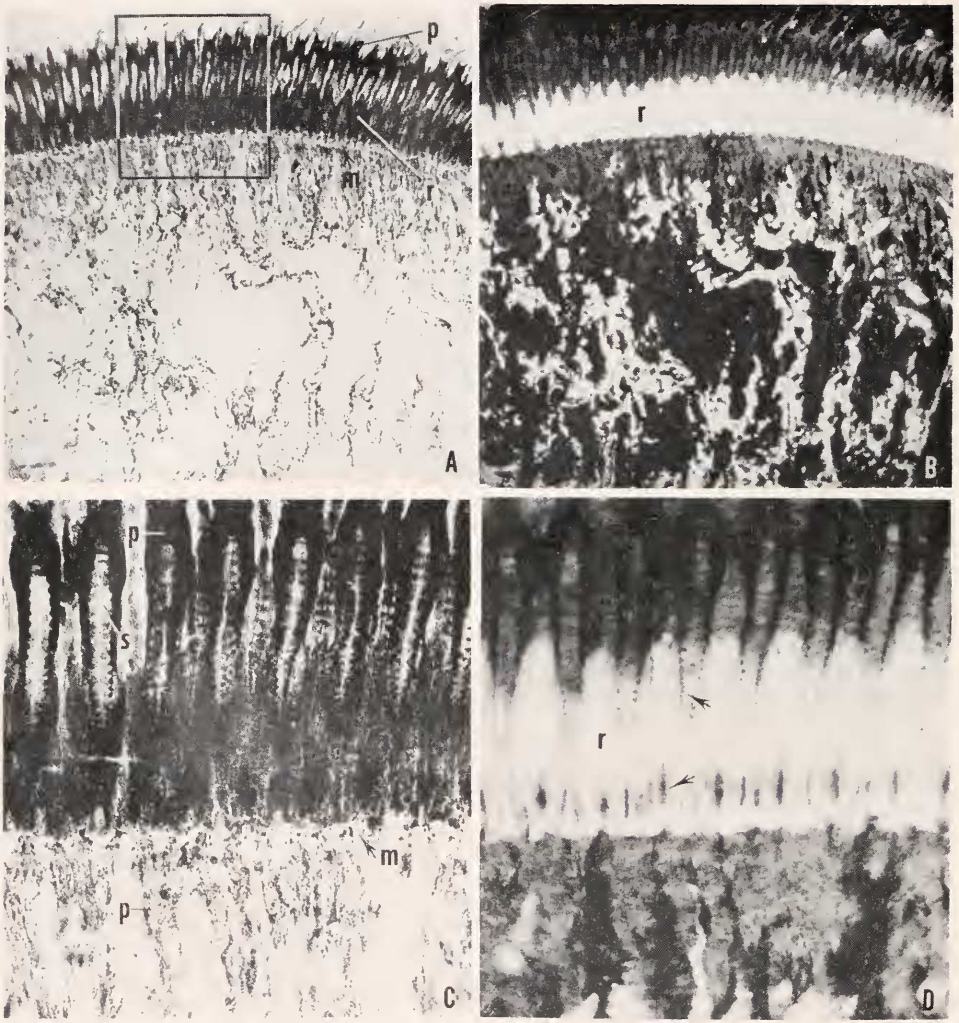

FIGURE 1. All the photographs are of a longitudinal section through the eye of *Homarus* and show the proximal portion of the retina. The bottom of each figure is proximal to the body; the top of the figure is distal from the body.

FIGURE 1A. Bright-field illumination; the proximal pigment and the layer of reflecting pigment above the fenestrated basement membrane surround the rhabdomes. Granules of both proximal pigment and reflecting pigment also occur below the basement membrane, but these are not readily distinguishable from each other.

FIGURE 1B. Dark-field illumination of the same region seen in Figure 1A. The layer of reflecting pigment distal to the fenestrated basement membrane, and the deposits of this pigment proximal to the basement membrane are now readily evident. Comparison of the distribution patterns of the pigments below the basement membrane in the two prints permits some differentiation between granules of reflecting pigment and of proximal pigment.

FIGURE 1C. The rectangular region marked in Figure 1A shown under higher magnification by bright-field illumination.

proximal to the basement membrane also were combined with the scrapings from the reflecting pigment layer.

In the case of *Limulus*, reflecting pigment is located distally in the eye. The intervening retinal melanin was exposed, chipped away with a small scalpel, and discarded. In a few instances most of this retinal melanin was dissolved by immersing the eye for an hour in ethylene chlorohydrin; the treated retinas were then washed in a few changes of ethanol. Either of these methods of removing the melanin exposed the reflecting pigment which was then scraped free and concentrated by centrifugation. Masses of white material, similar in appearance to the reflecting pigment, and described by some authors as "rudimentary eyes," are closely associated anatomically with the lateral and median eyes of *Limulus;* these, too, were removed for study.

The reflecting pigments and associated tissue were ground and extracted from 1 to 6 hours in a micro-centrifuge tube with 0.1 ml. per retina of one of the following alkaline solutions: 1% NaOH; 1% LiOH; 0.5 N NH_4OH; 0.06% Li_2CO_3; 0.2 *M* borate buffer at pH 9.2; or a solution of 50% ethanol containing 2% NH_4OH. The tubes were centrifuged and samples of the supernatant solution as well as samples of standard purine solutions were applied with a micro-pipette to sheets or strips of Whatman No. 1 filter paper for subsequent chromatography or electrophoresis.

Either ascending or descending development was used with a wide variety of solvent mixtures, such as are listed by Block, Durrum and Zweig (1955) and by Viscontini, Schmid and Hadorn (1955). The most useful solvent systems were: (1) water-saturated n-butanol:formic acid = 9:1; (2) pyridine:ethyl acetate:water = 4:3:3; (3) isoamyl alcohol saturated with 5% disodium hydrogen phosphate (with a layer of each in the chromatography chamber); (4) water-saturated collidine; (5) 3% aqueous ammonium chloride; (6) n-butanol:acetic acid:water = 8:2:2, followed by a second development in the same direction with acetone:n-butanol:water = 8:1:1. After development the paper was dried and examined in short-wave ultraviolet light (Mineralight Model V-41 lamp, manufactured by Ultraviolet Prod. Inc.) and the spots outlined with pencil. Tentative identifications of the components of the reflecting pigment were made by comparing the distances the component spots migrated with the distances migrated by the spots of reference standards. Spots developed from reflecting pigment were cut out and eluted in 0.1 N NaOH or 0.1 N HCl. The identification was then verified by determining the ultraviolet absorption spectra of these eluates in a Beckman spectrophotometer and comparing them with spectra of the known standards. In a large number of cases developed chromatograms were also treated according to the method of Vischer and Chargaff (1948) whereby purine spots are made visible as a black mercuric sulfide complex. The latter procedure revealed overlapping or masking of components when they occurred, and thus indicated need for development in different solvent systems.

Paper electrophoresis was used primarily in resolving one of the pteridines

FIGURE 1D. Dark-field illumination of the region shown in Figure 1C. Arrows point to granules of dark proximal pigment intermingled with the reflecting pigment layer. Strands of reflecting pigment at the bottom of the print aid in recognizing this pigment in Figure 1C. *m*, fenestrated basement membrane; *p*, proximal pigment; *r*, reflecting pigment; *s*, rhabdome.

which could not be satisfactorily separated from the other components of reflecting pigment by paper chromatography. Samples, about 0.1 ml. in volume, of ethanol-ammonia extract of lobster retina were applied to paper strips which were then developed at 375 volts or 500 volts for 18 to 20 hours in the LKB or the Spinco instrument. The buffer was 0.04 *M* boric acid and 0.01 *M* borax at pH 8.6. After the strips were developed and dried, the blue-fluorescent segments, which had migrated toward the cathode, were cut out and eluted in 0.1 N HCl or in 0.1 N NaOH for subsequent spectrophotometry.

Initial studies on solubility of the reflecting pigment of *Homarus* were made on histological sections cut at 10 microns from paraffin-embedded retinas. The mounted sections were de-waxed with xylene and re-hydrated before testing with the various solvents. The murexide and enzymatic tests were made on small amounts of reflecting pigment which had been removed as described. The methenamine-silver reaction of Gomori (1952) was used as a histochemical test for uric acid.

Results

A. Nature of the reflecting pigment of Homarus

Reflecting pigment was dissolved from sections of lobster retina within 30 minutes after immersion in 1 N solutions of specific acid (hydrochloric, acetic, nitric or sulfuric) or of specific alkali (ammonium hydroxide, sodium hydroxide, 0.1% aqueous solutions of sodium carbonate or sodium bicarbonate). At 60° C. the reflecting pigment dissolves within an hour in glycerine or ethylene glycol or ethylene glycol monoethyl ether. When, however, these same solvents are used at room temperature, one finds little visible solution in glycerine, partial solution in ethylene glycol monoethyl ether, and complete solution in ethylene glycol. The reflecting pigment is partially dissolved from sections remaining overnight in 95% ethanol but showed no discernible solution in absolute ethanol. These solubilities differ in several important respects from those reported for guanine by Millot (1923). Thus, according to Millot, guanine is insoluble in ammonium hydroxide or acetic acid, whereas the reflecting pigment of *Homarus* is soluble in both these solutions. Gwilliam (1950) also reports solubilities of retinal reflecting pigment of the crab, *Hemigrapsus oregonensis*, that fail to agree with those of guanine.

The residue obtained by evaporating to dryness a dilute lithium carbonate extract of *Homarus* reflecting pigment gives positive murexide but negative or faint, dubiously-positive Weidel reactions. Guanine, uric acid, xanthine and its methyl derivatives give positive murexide reactions (Lison, 1936). Millot (1923) reports that guanine and xanthine, but not uric acid, react positively to the Weidel test; adenine and hypoxanthine, among the other common purines, are reported to give neither murexide nor Weidel reactions. Comparison of these reported results with the findings for *Homarus* casts doubt on the reflecting pigment's being guanine and indicates, instead, that the reflecting pigment of the lobster may be uric acid.

A histochemical test depending on an argentaffin reaction between uric acid and methenamine-silver (Gomori, 1952) proved positive for the reflecting pigment of *Homarus*. Argentaffin reactions, particularly in neutral solution, have been criticized (Lison, 1936) because positive reactions are also given by calcium carbonate and phosphate, if present. In this study, however, exposure of sections to me-

thenamine during incubation is supposed to bring about ready solution of such calcifications.

More specific identification of uric acid in the reflecting pigment was made by paper chromatographic resolution of mixtures after incubation with uricase (Nutritional Biochemicals Corp.). Preliminary exploration showed that 1 to 5 μgm. of uric acid in 5 μl. of 0.5% lithium carbonate solution are detectable when the n-butanol-formic acid solvent system and the Vischer-Chargaff (1948) visualization method are used. Uric acid and 5 μl. of a solution containing the reflecting pigment of one lobster retina in 0.1 ml. showed similar R_f indices (0.14 to 0.17) with this same solvent system.

The reflecting pigment of 20 lobster eyes, dissolved in 0.5 ml. of dilute lithium carbonate solution, was mixed with 50 mg. of uricase, 0.5 ml. of 0.05 *M* borate buffer at pH 9.2, and 0.5 ml. of toluene. A 5-μl. sample of this mixture was removed for application to paper within 5 minutes (zero time). This mixture was gassed with oxygen and incubated at 38° C. Thereafter, at intervals of 0.5, 1, 2, 4, and 6 hours, 5-μl. aliquots were removed and applied to paper; a 5-μgm. sample of uric acid to serve as a reference standard was applied to the same sheet of paper which was then developed in butanol-formic acid solvent. Treatment of the developed chromatogram by the Vischer-Chargaff method revealed the purine as black spots with an R_f index of 0.15 for the reference standard and also for those aliquots taken at 0-, 0.5- and 1-hour intervals. The intensity of the spots decreased with time of incubation with uricase. The sample taken after 2 hours of incubation showed an R_f index of 0.14 and was very faint. No spots were present for the 4-hour and 6-hour samples. Because of the specificity of uricase in the oxidation of uric acid, these results may be considered a satisfactory demonstration of the presence of uric acid in the retinal reflecting pigment of *Homarus*. Examination of these chromatograms revealed an additional faint spot distal to each of the corresponding retinal uric acid spots; this faint spot was not present above the uric acid standard. The possible presence of other purines besides uric acid was indicated by this observation.

This possibility was explored by first examining chromatograms developed in butanol-formic acid solvent in ultraviolet light, and then using the mercuric nitrate-ammonium sulfide visualization method for purines. When this was done, the results diagrammed in Figure 2 were obtained for the lobster. The diagram shows the presence of three apparent purines, one of which is uric acid, and two fluorescent compounds. For subsequent reference, these spots are labelled, starting from the baseline on the chromatogram, as Fluorescent 1, Absorbent 1 (uric acid), Fluorescent 2, Absorbent 2, and Absorbent 3.

B. Further identification of the retinal compounds

The two fluorescent compounds of the reflecting pigment were believed to be pteridines which have been reported present in the eyes of vertebrates (Pirie and Simpson, 1946; Hama, 1953) and of crustaceans (Busnel and Drilhon, 1948). After chromatographic development of retinal pigment samples and aliquots of known purines and xanthopterin as reference standards in a variety of solvent systems, the R_f indices of the components were compared. In this way, four of the five spots of Figure 2 were identified: Absorbent 1 is uric acid; Fluorescent 2 is

xanthopterin; Absorbent 2 is xanthine; and Absorbent 3 is hypoxanthine. The linear sequence of the spots, starting from the baseline on the chromatogram, may vary strikingly with different solvent systems (Fig. 3). Advantage was taken of this property to make the final verification of the above-mentioned identifications. Well-resolved spots, not masked or overlapped by other components, were cut out, eluted in 0.1 N HCl, and the absorption spectrum of the eluate determined. The

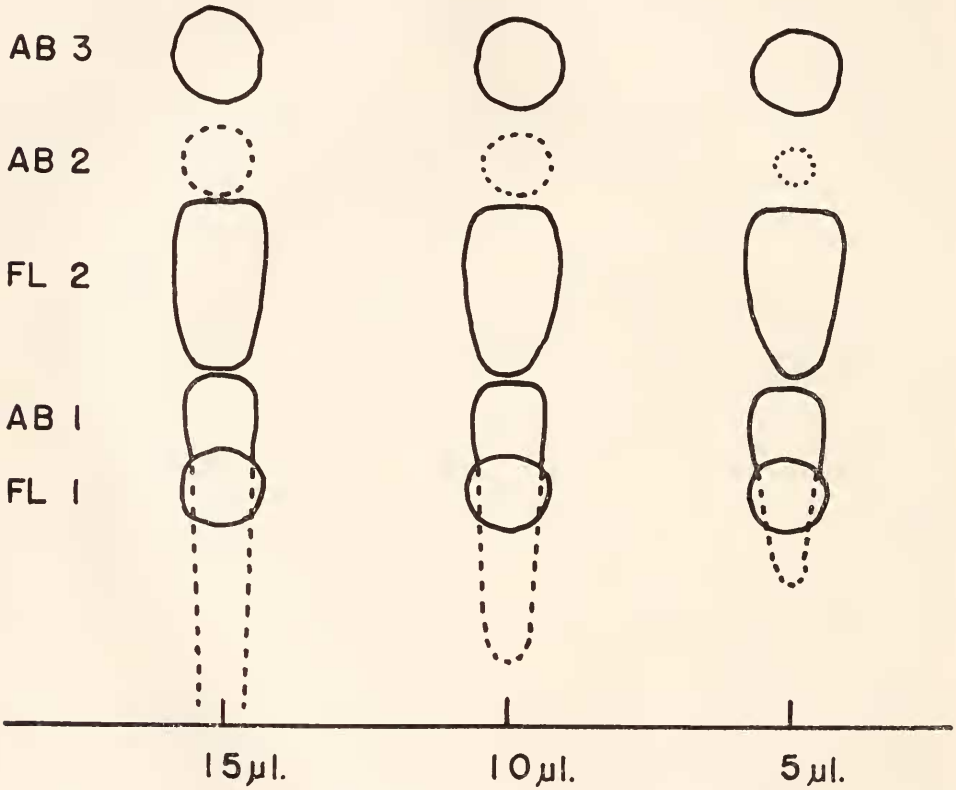

FIGURE 2. Tracing of a chromatogram of retinal reflecting pigment of *Homarus*, developed in butanol-formic acid solvent. The resolved spots were first outlined in ultraviolet light and then were treated to make the purine spots visible; the broken lines indicate boundaries made evident after this latter treatment. The size of the sample applied to the paper is given in microliters. The labels to the left identify and describe the appearance of the spots in ultraviolet light: FL, fluorescent; AB, absorbent. See text.

maxima of the spectra obtained for spots identified as uric acid, xanthine, and hypoxanthine corresponded with those reported by Dorough and Seaton (1954).

The absorption spectrum of the retinal component identified by R_f index as xanthopterin was determined after similar elution from a chromatogram developed in butanol-formic acid solvent. This is compared with the spectrum obtained from eluates of xanthopterin used as a reference standard on a paper chromatogram

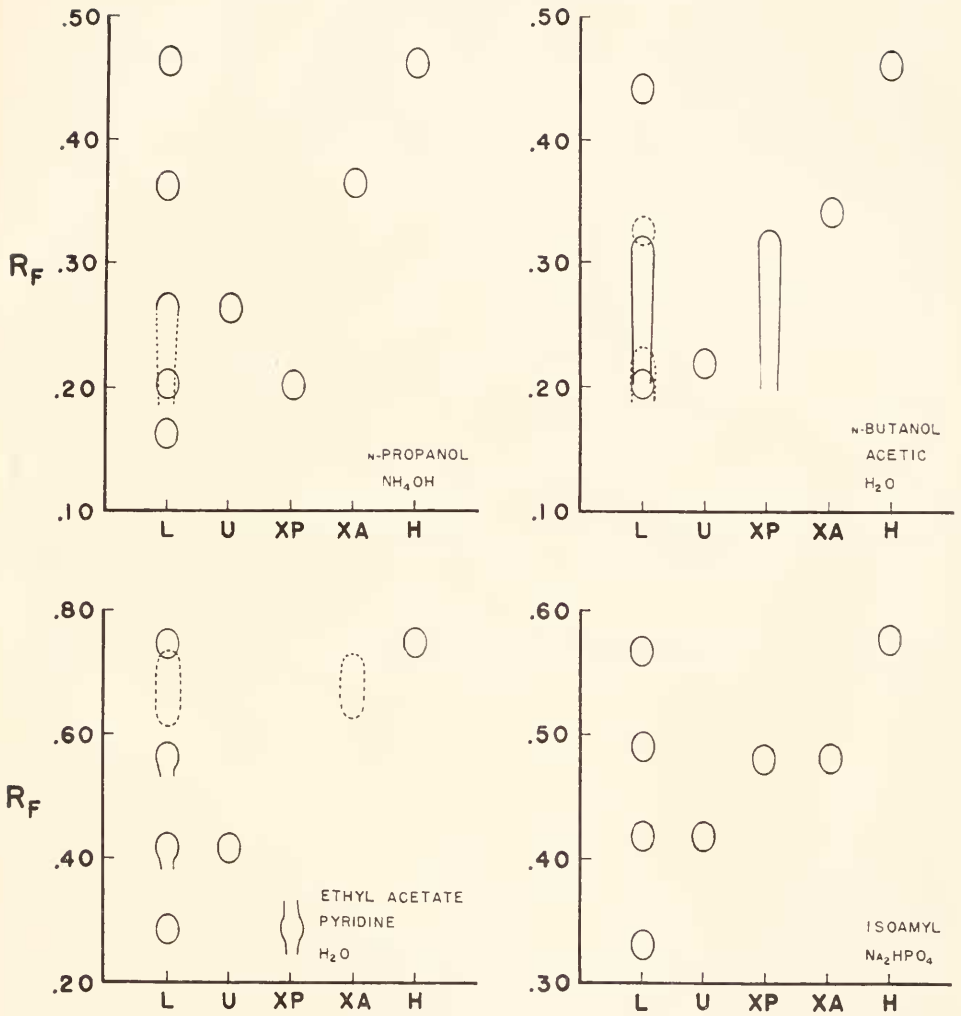

FIGURE 3. Diagrams showing the tentative identification of four of the five components of the reflecting pigment of *Homarus* arrived at by comparing the R_f indices of the components with those of reference standards. The solvent system is indicated on each diagram. Broken lines represent the boundaries of components not evident on examination in ultraviolet light but which became visible after formation of the mercuric sulfide complex. L, reflecting pigment of *Homarus;* U, uric acid; XP, xanthopterin; XA, xanthine; H, hypoxanthine.

(Fig. 4). The maxima of the reference xanthopterin at 230 mμ, 259 mμ, and 355 mμ agree with the maxima reported for xanthopterin by Elion and Hitchings (1947). The spectrum of xanthopterin from the retina shows similar maxima at 231 mμ, 261 mμ, and 355 mμ, although the geometry of the retinal spectrum differs somewhat from that of the reference standard. The basis of this difference is not understood.

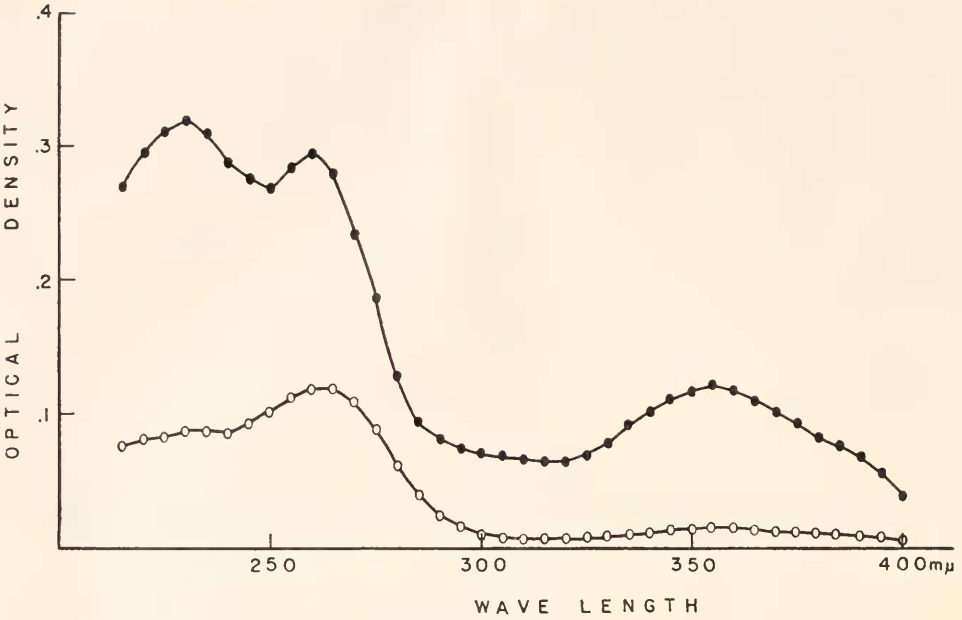

FIGURE 4. Absorption spectra of reference xanthopterin (upper curve) eluted from paper chromatogram and of Fluorescent 2 component (lower curve) from reflecting pigment of *Homarus*.

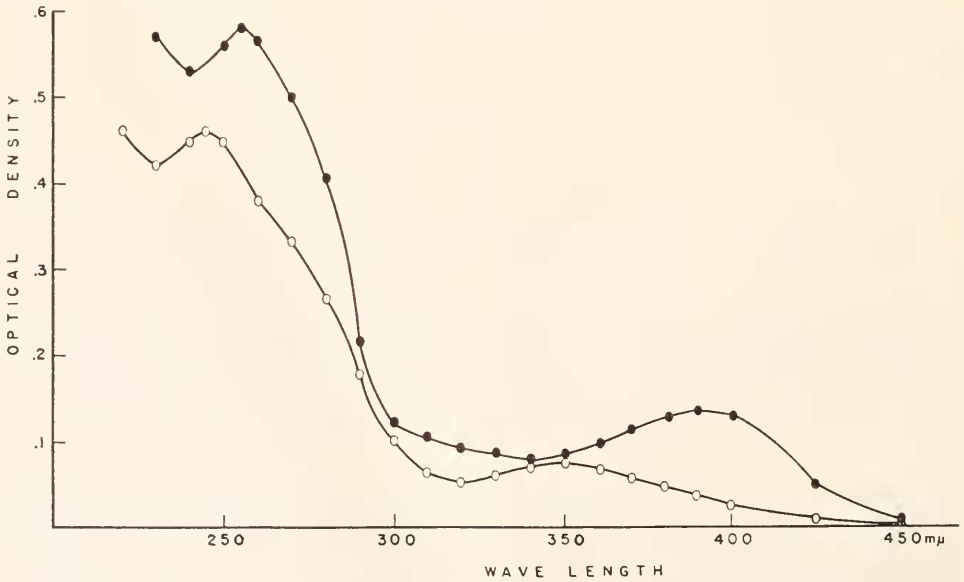

FIGURE 5. Absorption spectra of unidentified Fluorescent 1 component of lobster reflecting pigment. Upper curve is the eluate from the paper in 0.1 N NaOH; lower curve is the eluate in 0.1 N HCl.

C. The unidentified fluorescent component

There remains to be considered the unidentified component of *Homarus* reflecting pigment labelled Fluorescent 1 in Figure 2. The rarity of many pure pteridines limited the number available for use as chromatographic standards; the R_f indices of the few pteridines used for this purpose failed to give a satisfactory match with the index for Fluorescent 1 in a variety of solvent systems. Attempts to isolate this component in sufficient concentration for subsequent spectrophotometry, as was done with the other retinal components, were generally frustrated by contamination due to streaking or tailing of the other constituents.

Fluorescent 1 was finally isolated by paper electrophoresis, and was eluted in 0.1 N HCl or in 0.1 N NaOH, as described in Methods. The absorption spectrum of the eluate in acid showed maxima at 245 mμ and 353 mμ; in alkali, these maxima were shifted to 255 mμ and 390 mμ (Fig. 5).

D. Specific localization within the retina

It cannot be stated with complete certainty in which part of the lobster retina the five purines and pteridines are specifically localized. The evidence described above indicates that uric acid is most probably a component of the reflecting layer of retinal pigment, as may also be the two other purines, xanthine and hypoxanthine. The two pteridines may be more widely distributed among the retinal components. Busnel and Drilhon (1948) found several substances, detectable by fluorescence microscopy, in the crustacean retina. These fluorescent materials not only are closely associated with the proximal pigment but also occur in the regions of the reflecting and distal pigments.

It is apparent from Figure 1 (C and D) that, although most of the proximal pigment in light-adapted retinas has migrated distal to the reflecting pigment layer, proximal pigment granules still remain intermingled with and below this layer. The preparation of reflecting pigment for chromatography unavoidably included some of these proximal pigment granules. However, chromatography of preparations of reflecting pigment, previously washed with ethylene chlorohydrin to remove the traces of dark proximal pigment, showed the presence of the two pteridines obtained with untreated reflecting pigment. Thus, while the above observations are presumptive evidence for localization of the pteridines in the reflecting pigment, the possibility of their occurring also in the other retinal pigments cannot be excluded.

E. Retinal reflecting pigment in Limulus

Reflecting pigment from the lateral and median eyes of *Limulus* was obtained as described under Methods. The deposits of white material of so-called rudimentary eyes, located in the postero-medial region of each lateral eye, as well as similar material associated with the median eyes, were dissected free. Each of these was dissolved separately in 0.5% NaOH. Samples of the solutions were applied to paper and developed, along with a series of purine reference standards. The solvent systems were butanol-formic acid; water-saturated collidine; and butanol-water-morpholine-diethylene glycol. Examination of the chromatogram in ultraviolet light generally revealed a single quenching spot whose R_f index was the same as that

of guanine. A faintly bluish-fluorescing spot was also evident in one case but was not observable in any of the other chromatograms. Chromatograms treated by the Vischer-Chargaff method confirm the coincidence of R_f indices for the reference guanine and reflecting pigment from lateral, median, and rudimentary eyes.

The spots quenching ultraviolet light, obtained with reflecting pigment from a lateral eye, were cut from a chromatogram developed in butanol-formic acid and were eluted overnight in 1% NaOH. The spectrum of this eluate had a maximum at 275 mμ, in agreement with that reported by Hotchkiss (1948) for guanine.

I am indebted to Profs. C. M. Williams and J. H. Welsh for helpful suggestions and critical comments on the manuscript.

Summary

1. The chemical nature of the retinal reflecting pigment was studied in *Homarus* and in *Limulus*. In crustaceans the reflecting pigment has been thought to be guanine, but the solubility and chemical properties of this pigment from *Homarus* do not agree with those for guanine.

2. Use of paper chromatographic methods shows the presence of five substances in the reflecting pigment of *Homarus,* three of which are absorbent or quenching in ultraviolet light and two of which are fluorescent.

3. Histochemical treatment with methenamine-silver and incubation studies with uricase identify one of the three ultraviolet-absorbent compounds as uric acid. Comparisons of R_f indices of the other two ultraviolet-absorbent compounds with those of reference purines show them to be xanthine and hypoxanthine. Identifications of all three were verified by determining the ultraviolet absorption spectra of the retinal purines eluted from paper chromatograms.

4. One of the two fluorescent components of *Homarus* reflecting pigments is xanthopterin, identified both by its R_f indices after chromatographic development in a variety of solvent systems, and by its absorption spectrum. The second fluorescent compound, probably a pteridine, has not been identified, but its absorption spectrum shows maxima at 245 mμ and 353 mμ in 0.1 N HCl; in alkali these maxima are shifted to 255 mμ and 390 mμ.

5. Retinal reflecting pigment from *Limulus* is guanine.

LITERATURE CITED

Block, R. J., E. L. Durrum and G. Zweig, 1955. A Manual of Paper Chromatography and Paper Electrophoresis. Academic Press Inc. New York. 484 pp.

Brown, F. A., Jr., M. N. Hines and M. Fingerman, 1952. Hormonal regulation of the distal retinal pigment of *Palaemonetes. Biol. Bull.,* **102**: 212–225.

Brown, F. A., Jr., H. M. Webb and M. I. Sandeen, 1953. Differential production of two retinal pigment hormones in *Palaemonetes* by light flashes. *J. Cell. Comp. Physiol.,* **41**: 123–144.

Busnel, R. G., and A. Drilhon, 1948. Sur les pigments flaviniques et ptériniques des Crustacés. *Bull. soc. zool. France,* **73**: 142–185.

Dorough, G. D., and D. L. Seaton, 1954. A method for the extraction and assay of nucleic acid fragments in tissues. *J. Amer. Chem. Soc.,* **76**: 2873–2877.

Elion, G. B., and G. H. Hitchings, 1947. The synthesis of some new pteridines. *J. Amer. Chem. Soc.,* **69**: 2553–2555.

Gomori, G., 1952. Microscopic Histochemistry. Univ. of Chicago Press. Chicago. 273 pp.

GWILLIAM, G. F., 1950. On the occurrence and solubility of a reflecting pigment in the eyes of the Brachyura. *Anat. Record,* **108**: 613.

HAMA, T., 1953. Substances fluorescentes du type ptérinique dans la peau ou les yeux de la grenouille (*Rana nigromaculata*) et leurs transformations photochimiques. *Experientia,* **9**: 299–300.

HOTCHKISS, R. D., 1948. The quantitative separation of purines, pyrimidines, and nucleosides by paper chromatography. *J. Biol. Chem.,* **175**: 315–332.

KLEINHOLZ, L. H., 1936. Crustacean eye-stalk hormone and retinal pigment migration. *Biol. Bull.,* **70**: 159–184.

KLEINHOLZ, L. H., 1955. The nature of the reflecting pigment in the arthropod eye. *Biol. Bull.,* **109**: 362.

LISON, L., 1936. Histochemie Animale. Gauthier-Villars. Paris. 320 pp.

MILLOT, J., 1923. Le pigment purique chez les vertébrés inférieurs. *Bull. Biol. France et Belg.,* **57**: 261–363.

PIRIE, A., AND D. W. SIMPSON, 1946. Preparation of a fluorescent substance from the eye of the dogfish, *Squalus acanthias. Biochem. J.,* **40**: 14–20.

VISCHER, E., AND E. CHARGAFF, 1948. The separation and quantitative estimation of purines and pyrimidines in minute amounts. *J. Biol. Chem.,* **176**: 703–714.

VISCONTINI, M., H. SCHMID AND E. HADORN, 1955. Isolierung fluoreszierender Stoffe aus *Astacus fluviatilis. Experientia,* **11**: 390–392.

WELSH, J. H., 1932. The nature and movement of the reflecting pigment in the eyes of crustaceans. *J. Exp. Zool.,* **62**: 173–183.

WELSH, J. H., 1939. The action of eye-stalk extracts on retinal pigment migration in the crayfish, *Cambarus bartoni. Biol. Bull.,* **77**: 119–125.